Ubiquitin in Motor Neuron Disease: Study at the Light and Electron Microscope

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Abstract. Several neurodegenerative diseases, including motor neuron disease (MND), are characterized by formation of abnormal cytoskeleton-derived inclusions which contain ubiquitin (Ubq). We have studied the distribution of Ubq in 26 cases of MND with light and electron microscopic immunocytochemistry. Ubiquitin-positive inclusions were found in neurons of anterior horns in most cases of amyotrophic lateral sclerosis (ALS) but were not present in other forms of MND. Ubiquitin immunoreactivity was observed in 10–15 nm intraneuronal filaments, which were not stained by antibodies to neurofilaments, and on dense bodies of dystrophic neurites throughout the neuripil of anterior horns and pyramidal tracts. Data analysis showed a trend toward lower percentage of Ubq-positive neurons in cases with longer duration of illness or lower number of neurons. A high percentage of Ubq-positive inclusions occurred in cases with an aggressive clinical course, suggesting that ubiquitination takes place at early stages of the disease.

Key Words: Immunoelectron microscopy; Immunohistochemistry; Motor neuron disease; Neurofilaments; Ubiquitin.

INTRODUCTION

Most neurodegenerative diseases are characterized by the occurrence in affected neurons of inclusion bodies, which may be membrane-bound or non-membrane-bound. The former are mainly represented by lysosomal bodies or by bodies formed in the rough endoplasmic reticulum. The latter often contain cytoskeletal proteins and share the component ubiquitin (Ubq) (1).

Ubiquitin is a 76 amino acid protein which is present in cells as a free molecule or conjugated to selected proteins (2). Some Ubq-conjugated proteins are targeted for rapid degradation through an ATP-dependent, non-lysosomal proteolytic system, while others are more stable (3). Candidates for Ubq-mediated rapid proteolysis are abnormal and normal short-lived proteins. Among more stable conjugates are some histones, membrane receptors and microtubule-associated proteins (2, 4).

Besides neurofibrillary tangles in Alzheimer’s disease (5–10), Lewy bodies in Parkinson’s disease (8–13), and Pick bodies (8–10), Ubq-positive inclusions can be found in spinal motor neurons of patients with motor neuron disease (MND), where they have been suggested to represent a hallmark (14–20). Formation of Ubq-positive inclusions is thought to result from the accumulation of damaged proteins which would form during prolonged cell stress (10). The excessive amount of abnormal proteins would thus saturate the Ubq-mediated proteolytic system (10). However,
other mechanisms may be involved: deposits could represent stable high molecular weight Ubq-conjugates not targeted for proteolysis (3), or ubiquitinated cytoskeletal components (4); moreover, ubiquitinated substrates may become cross-linked to other proteins (3). Recent evidence suggests that proteins may be targeted by Ubq for lysosomal-mediated proteolysis (21). The nature of the Ubq-positive inclusions in MND and their role in the pathogenesis of the disease remain unclear (22). In this paper, we examine the light and electron microscopic localization of Ubq epitopes in a series of 26 cases of MND.

MATERIALS AND METHODS

Twenty cases of amyotrophic lateral sclerosis (ALS), two cases of hereditary progressive muscular atrophy (hPMA) (23), two cases of sporadic PMA (sPMA), one case of progressive bulbar palsy (PBP), one case of Werdnig-Hoffmann's disease (WH) and six controls were studied. Of the 20 ALS cases, 13 were males and 7 were females. Patient age ranged from 43 to 72 years with a mean of 59.8 years (±8.7). The duration of illness ranged from 6 to 60 months, with a mean of 31.5 months (±19.27). The hPMA cases were a 30 year old male and a 56 year old female with 42 and 6 months duration of illness, respectively. The sPMA cases were a 61 year old male and a 69 year old female with 48 and 18 months duration of illness, respectively. The PBP case was a 27 year old female, with a duration of illness of 12 months.

Control cases were affected by non-degenerative neurologic diseases of the spinal cord: two cases of spinal angioma, one case of multiple sclerosis, one case of spinal astrocytoma, one case of ischemia and one case of transverse myelitis. Their age ranged from 36 to 62 years, with a mean of 55.2 years (±7.3).

Brains and spinal cords obtained at autopsy were fixed in 10% formalin, and specimens of the following areas were embedded in paraffin: motor cortex; midbrain; pons; medulla; cervical, dorsal and lumbar spinal cord. Five µm serial sections were cut and stained with Hematoxylin-Eosin, Luxol Fast Blue B, Cresyl violet, Bodian and PAS.

Immunohistochemistry was performed with the peroxidase anti-peroxidase (PAP) method using nine antibodies (Ab): (a) rabbit antiserum to glial fibrillary acidic protein (GFAP) (Dako, Santa Barbara, CA; diluted 1/800); (b) monoclonal antibody to vimentin (Dako, diluted 1/20); (c) a battery of monoclonal Ab to neurofilaments (NF): anti-68, -160, -200 Kd subunits (Boehringer, Indianapolis, IN; diluted 1/5), SMI-31 (to phosphorylated epitopes) and SMI-32 (to non-phosphorylated epitopes) (Sternberger, diluted 1/500); (d) an affinity-purified rabbit antiserum to Ubq, which recognizes both free and conjugated Ubq (10) (diluted 1/800); (e) a monoclonal antibody to microtubule-associated tau protein (24). Immunogold electron microscopy for Ubq and NF was performed on spinal cord of five cases of ALS. Two methods were used (20): 1) pre-embedding staining on Vibratome sections or on sections cut from paraffin blocks; and 2) post-embedding staining on tissue blocks embedded in London Resin White.

Motor neurons of both anterior horns, which were defined as the gray matter anterior to a horizontal line originating from the central canal, were counted in three consecutive sections of cervical and lumbar segments of spinal cord stained with Cresyl violet. Ubiquitin-positive motor neurons were counted in three adjacent serial sections. The number of motor neurons and of Ubq-positive motor neurons was calculated as the mean value between cervical and lumbar sections. Correlations were calculated by means of the least squares method (25).

RESULTS

Ubiquitin-positive inclusions were found in 16 of the 20 cases of ALS. They were located in the neurons of the anterior horns at either or both the cervical and lumbar levels in all 16 positive cases. They were also observed often in the nucleus of the XII cranial nerve and occasionally in the nuclei of the IX and X cranial nerves. No positivity for Ubq was found in the spinal cord of PMA, WH and PBP cases nor in

the six controls; also, spheroids were negative. Diffuse immunostaining with antibody to Ubq was present in ballooned cells in medulla and pons of the WH case and in neurons in the nucleus of the XII cranial nerve of the PBP case. No staining was seen in neurons of motor cortex.

The intracellular Ubq-positive structures or inclusions present in motor neurons were classified as: (a) skein-like or filamentous structures in perinuclear or peripheral position (Fig. 1A); (b) large, dense ball-like deposits with granular or filamentous profiles (Fig. 1B and C); (c) circumscribed structures composed of granules also associated with vacuoles (Fig. 1D); (d) mixed forms (Fig. 1E); (e) in some neurons a granulo-filamentous staining was observed in the proximal tract of axons (Fig. 1F); (f) in one case, round inclusions with a more intense peripheral ring, resembling Lewy bodies of Parkinson's disease, were present (Fig. 1G); and (g) spheroids of anterior horns and pyramidal tracts.

Other Ubq-positive structures were found in anterior horns and pyramidal tracts and were represented by dot-like stainings (Fig. 2).

With immunoelectron microscopy, the skein-like inclusions were found to correspond to bundles of 10–15 nm filaments, which were often adjacent to unlabeled lipofuscin granules (Fig. 3a). The ball-like structures could be identified as large inclusions composed of 15–20 nm filaments, whereas adjacent normal 8–10 nm filaments were unlabeled (Fig. 3b). Gold labeling corresponding to the dot-like Ubq-positive structures was seen. In pyramidal tracts and in the neuropil of the anterior horns, gold labeling was found on amorphous densities inside small axons (Fig. 4a) and, occasionally, in intra- or juxtamyelinic structures, probably corresponding to cytoplasm of oligodendrocytes (Fig. 4b).

All the antibodies to NF stained axons, axonal swellings and spheroids. The reaction was most intense with SMI-31. Diffuse or partial staining of some perikarya was seen mainly with SMI-31; however, no intraneuronal staining pattern similar to that obtained with antiserum to Ubq could be demonstrated, either in the light or in the electron microscope.

Staining for tau protein was negative. GFAP and vimentin were positive in reactive astrocytes of anterior horns and pyramidal tracts.

**Cell Counting:** The mean number of motor neurons per section in ALS cases ranged from 27 ± 6 to 100 ± 12. There was no statistically significant correlation between mean number of neurons and either age or duration of illness. The percentage of ubiquitinated neurons ranged from 0 to 20.6%. No significant correlation was found between percentage of ubiquitinated neurons and age (Fig. 5A), duration of the disease (Fig. 5B) or number of neurons (Fig. 5C); however, there appeared to be a trend toward a lower percentage in older patients and in cases with longer duration, as shown by the finding that the cases with a high percentage of ubiquitinated neurons had the shortest duration of illness and the lowest number of neurons.

**DISCUSSION**

The majority of the ALS cases showed Ubq-positive inclusions in lower motor neurons, in accordance with previously reported data (14, 15, 19, 26). This finding is even more striking considering that Ubq-containing inclusions were not present in the other forms of MND, i.e. sPMA, hPMA, PBP and WH. The term MND has been used because of the difficulty in distinguishing, at least in the early stages, ALS from PMA, PBP and primary lateral sclerosis (PLS) (27). PMA is usually a slow, progressive disease. It could represent either the initial stage of ALS or develop into PBP. Amyotrophic lateral sclerosis has a fatal outcome in 50% of patients within
three years, but may also progress slowly (28, 29). On the other hand, the ratio of PMA:ALS is known to be lower if the diagnosis is made on neuropathological findings (30). The positive staining for Ubq in the medulla and not in the spinal cord of the PBP case could be in line with the inclusion of PBP in the MND group.

The four ALS cases negative for Ubq have different possible explanations. In three cases, the absence of Ubq-positive neurons might be due to the severe loss of neurons associated with a long duration of illness. One of these three cases was a patient with bulbar symptoms who died shortly after the appearance of spinal signs; this case might also have been classified as a bulbo-spinal form instead of ALS. On the other hand, one of the ALS cases with a high number of ubiquitinated neurons showed neither clinical nor pathological signs of involvement of pyramidal tracts. Also, this case might be an ALS case in which the disease had not yet progressed to involve the pyramidal tracts, or it might be a bulbo-spinal form. Although four out of 20 cases of ALS showed no Ubq-positive inclusions, it is clear that overall ALS cases are characterized by ubiquitinated neurons, while other MND cases are not. The lack of Ubq in motor neurons of PMA might indicate a different degenerative process in comparison with ALS. However, the difficulty in attributing some cases to one or the other group suggests caution in distinguishing ALS and PMA on the basis of Ubq immunostaining.

Different observations have been made in WH: ballooned cells with diffuse Ubq-positivity have been found in anterior horns, Clarke's column and dorsal root ganglia.

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**Fig. 1.** Patterns of ubiquitin (UBq) immunostaining in spinal motor neurons. A: Skein-like deposits. ×1,000. B: Dense ball-like deposits with granular appearance. ×400. C: Dense deposits with filamentous appearance. ×1,000. D: Granular deposits associated with vacuoles. ×1,000. E: Deposits of mixed forms. ×400. F: Corkscrew-like staining in the proximal part of an axon. ×400. G: Staining of a Lewy body-like structure. ×1,000.
Fig. 3. Ubq immunoelectron microscopy. (a) Gold labeling for Ubq in intraneuronal bundles of 10–15 nm filaments, 10 nm gold granules. ×60,000. (b) Gold labeling for Ubq in 15–20 nm filamentous structures of large round deposits; adjacent normal neurofilaments are
Fig. 4. Ubq immunoelectron microscopy. (a) Small dystrophic axons with labeled amorphous materials. ×55,000. (b) Labeled intramyelinic clefts. ×60,000.

(17), and also in thalamus (31), but not in motor nuclei of the brain stem (17). On the contrary, Kato and Hirano (32) found ballooned cells with ubiquitinated vesicles and granules in extraocular muscle nuclei of the midbrain. In our case the occurrence of Ubq-positive ballooned cells was limited to the brain stem, and the staining showed a diffuse pattern. The common characteristic of all these observations is that the aspect of the Ubq-positivity is different from that found in ALS.

Although no statistically significant correlation between the percentage of Ubq-positive neurons and the age of patients or the duration of illness was found, a trend toward reduced immunoreactivity both in advanced ages and in long clinical courses was observed. Our data suggest that activation of the Ubq-dependent proteolytic system is not related to neuronal death but rather to the presence of a large quantity of abnormal proteins formed in neurons at relatively early stages of the disease. The trend toward a correlation between high percentage of Ubq-reactive neurons, short duration of illness and low number of neurons suggests that formation of Ubq-containing inclusions is related to the aggressiveness of the disease.

The main question regarding Ubq-positive structures is the nature of their constituents. Our data confirm that spheroids and axonal swellings, which contain Ubq epitopes, are formed by accumulations of NF, as already demonstrated by electron microscopy (EM) (33) and immunohistochemistry (34). On the contrary, a yet unresolved problem is the nature of the intraneuronal Ubq-positive inclusions.

not labeled, 20 nm gold granules. ×24,000. Inset: higher power view of labeled filamentous structures. ×50,000.
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In accordance with previous findings obtained with diaminobenzidin (DAB) (15), we have shown by ultrastructural immunogold labeling that intraneuronal Ubq-positive deposits correspond to filamentous structures of 10–15 nm (20). It is known that formalin fixation is responsible for reduction or absence of immunoreactivity to NF antibodies in neuronal perikarya (35); however, using a library of monoclonal antibodies to phosphorylated and non-phosphorylated NF, positive structures can be found not only as spheroids and axonal swellings, but also as diffuse intraneuronal staining, both in ALS and, less frequently, in controls (36–41). Similar data have been obtained by us; however, neither present nor previous observations (14–16, 20) have shown NF-positive intraneuronal structures corresponding to those stained for Ubq. It is possible that Ubq-positive deposits are formed by NF altered by the ubiquitination process and, therefore, are undetectable by antibodies; alternatively, abnormally formed NF might undergo ubiquitination, or NF are not components of the Ubq-positive filaments. The possibility that formalin fixation specifically reduced the NF immunogenicity of Ubq-positive inclusions cannot be ruled out. The observation that these structures are always found intracellularly may indicate that, differently from what is observed in other neurodegenerative diseases, they are not resistant to proteases after neuronal death.

In one of the sporadic cases of ALS, the intraneuronal Ubq reaction was globular and resembled Lewy bodies, which have been described both in sporadic (16) and familial (18) ALS. In the sporadic case the bodies were stained for Ubq in the halo and were negative for NF; in the familial cases Ubq was reported positive in the core, whereas NF stained only in the halo. Our finding is similar to that of Kato et al (16); it cannot be established whether the two different staining patterns refer to different pathogeneses.

Finally, the presence of dot-like immunostaining in anterior horns and pyramidal tracts might be attributed to “dystrophic” neurites, both for their immunohistochemical and ultrastructural aspects. They probably correspond to similar structures seen around senile plaques of Alzheimer’s disease (6, 42) and of Gerstmann-Sträussler-Scheinker disease (43), as well as in normal aging brain (44, 45). In ALS, they may represent a distal response of the axon to the neuronal degeneration.

REFERENCES


Fig. 5. Correlation between percentage of ubiquitinated neurons and Top: Age of patients ($r^2 = 0.12; p = n.s.$). Middle: Survival ($r^2 = 0.18; p = n.s.$). Bottom: Mean number of residual motor neurons ($r^2 = 0.02; p = n.s.$).

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